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Date: August 25, 2003

By: Kay L. Gaviglio

PATENT
Docket No. GCL266-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application: F. Valle et al.	)	
Serial No.: 08/940,692	)	APPEAL NO.
Filed: September 30, 1997	)	
Examiner: R. Prouty, Ph. D.	)	
Group Art Unit: 1652	)	

For: APPLICATION FOR GLUCOSE TRANSPORT MUTANTS FOR PRODUCTION OF AROMATIC PATHWAY COMPOUNDS

APPELLANTS' BRIEF ON APPEAL

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to 37 C.F.R. §§1.191 - 1.192, appellants file this Brief in support of the Notice of Appeal, dated June 24, 2003. Appellants respectfully submit, in view of the facts, arguments and authorities set forth below, the Board should find the final rejection of pending claims 23 - 27, 29 - 31, 33 - 40, 42 - 44 and 46 - 50 to be in error and should reverse.

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1. REAL PARTY IN INTEREST

Pursuant to 37 C.F.R. §1.192(c)(1), appellants Fernando Valle et al. declare Genencor International, Inc. is the real party in interest in this appeal.

2. STATEMENT OF RELATED CASES

Pursuant to 37 C.F.R. §1.192(c)(2), counsel for appellants state that this appeal was never previously before the Board of Patent Appeals and Interferences for final hearing. There are no presently pending related appeals or interferences, or appeals, which were decided by the Board, the Court of Appeals for the Federal Circuit, or a district court under 35 U.S.C. §146.

3. STATUS OF THE CLAIMS

The instant application was filed on September 30, 1997 as a file wrapper continuation application, of application serial number 08/435,510 filed May 5, 1995. Twenty-two claims were originally filed in the application.

In response to a first action on the merits, appellants canceled original claims 1 - 22 and submitted new claims 23 - 39. Claims 23 - 39 were finally rejected in an office action dated September 25, 1998.

A Notice of Abandonment dated April 27, 1999 was received by appellants for failure to file a timely response to the office action dated September 25, 1998. Appellants filed a petition on October 12, 1999 under 37 CFR 1.137(b) for revival of an unintentionally abandoned application. The petition was accompanied by a Notice of Appeal and an amendment under 37 CFR 1.116, wherein claims 40 - 46 were added and claim 32 was canceled. In an advisory action dated December 2, 1999, appellants were notified that the rejection of claims 23 - 39 was maintained, and that the amendment filed October 12, 1999 would not be entered.

A request was filed on April 10, 2000, for a Continued Prosecution Application (CPA) accompanied with a request to enter the amendment filed October 12, 1999. Upon entry of the amendment, claims 23 - 31 and 33 - 46 were pending. Further amendments were filed on July 10, 2000 and May 31, 2001, wherein claims 23, 25, 26, 27, 33, 34, 36, 38, 39, 43 and 44 were amended.

In response to the communications filed July 10, 2000 and May 31, 2001, a final office action dated July 25, 2001, was received. The rejection of claims 23 - 31, 33 - 38, 41, 42 and 44 - 46 was maintained, and claims 39, 40 and 43 were indicated as allowable.

In response to the final rejection, a request for a second Continued Prosecution Application (CPA) was filed on January 17, 2002. A first action final rejection dated August 13, 2002 indicated claims 23 - 31, 33 - 38, 41, 42 and 44 - 46 remained rejected, and claims 39, 40 and 43 were objected to but indicated as allowable. In response to the final rejection, a request for continued examination (RCE) was filed on December 13, 2002 with an accompanying amendment canceling claims 28 and 41 and adding new claims 47 - 51.

A final office action dated March 26, 2003 rejected claims 23 - 27, 29 - 31, 33 - 40 and 42 - 50 and indicated claims 39, 40, 43, 47, 48 and 51 were allowed. No further amendments were submitted, and a Notice of Appeal dated June 24, 2003 was filed contesting the rejection of claims 23 - 27, 29 - 31, 33 - 40 and 42 - 50.

Appellants have concurrently submitted, with the instant Appeal Brief, a further amendment canceling claim 45. Therefore, with entry of the amendment, claims 23 - 27, 29 - 31, 33 - 40, 42 - 44 and 46 - 50 are the claims on appeal. Rejected claims 23, 27, 38, 40 and 42 are independent claims. A copy of the pending rejected claims is attached hereto as Appendix 1. Appellants have also submitted, as Appendix 2, a copy of the allowed claims.

4. STATUS OF THE AMENDMENTS

As stated above in Section 3, appellants have concurrently submitted an amendment to the claims, wherein claim 45 has been canceled as being redundant of step (b) of independent claim 38. Further claim 46 has been modified to depend from claim 38 as opposed to now canceled claim 45. There have been no further amendments submitted in response to the final rejection dated March 26, 2003.

5. SUMMARY OF THE INVENTION

In order to facilitate a better understanding of the invention and for the Board's convenience, appellants have provided some general background regarding the biochemistry of

glucose metabolism in mesophilic bacteria. Reference is made to pages 7 - 9 of the disclosure and to modified Figure 1 as provided in Appendix 3.

Numerous references teach that in many bacteria, but not all bacteria, phosphoenolpyruvate (PEP) is necessary for transport of glucose into the microbial cell and that glucose is phosphorylated in a concerted process by a multiprotein membrane bound complex termed the phosphotransferase system (PTS). The PTS comprises the gene *pts*H, which encodes enzyme I; the gene *pts*I, which encodes the protein Hpr; and the gene *crr*, which encodes soluble glucose specific enzyme IIA. In this PTS process, PEP serves as a source of energy, and the phosphate from PEP is used in the conversion of glucose to yield glucose-6-phosphate and pyruvate.

PEP is also used as an intermediate for the production of many desired products; for example, (i) aromatic amino acids, such as phenylalaine, tyrosine and tryptophan and (ii) for the formation of oxaloacetate, which serves as a backbone for products, such as citrate and the amino acid aspartate. However, a large percent of the PEP produced, over 50%, is used to provide the energy for glucose uptake. Thus, as a result, the amount of PEP available for the biosynthesis of other compounds, such as oxaloacetate is severely reduced. (See pages 2 - 3 of the disclosure). Ancillary mechanisms exist in PTS strains, which are involved in glucose transport, but some of these transport mechanisms are inhibited or repressed from functioning under normal PTS conditions. One such system is the proton-linked transport protein, galactose permease (GalP). (See page 513, col. 2 of the Saier reference attached as Appendix 4 and cited herein under Section 8.A. Analysis of the references; pages 9 - 10 of the disclosure).

The present invention provides one means of solving the problem of reduced PEP availability for the biosynthetic production of desired products in a host cell which normally utilize the PEP:PTS and which includes an endogenous galactose permease. (See pages 9, lines 31 - 31, 2nd paragraph of the disclosure). More specifically, the PTS system, which utilizes PEP to transport glucose, is inactivated resulting in a PTS^- phenotype. Strains which are PTS^- are not very efficient at utilizing glucose and are also referred to as having a PTS^-/ Glu^- phenotype.

In the present invention, the PTS^-/ Glu^- strains are cultured on glucose as the sole carbon source and mutant strains (PTS^-/ Glu^+) are selected whereby the strains are capable of efficiently transporting glucose by a non-PTS mechanism and having a specific growth rate of at least 0.4 hr^{-1}. (See page 5, lines 15 - 22 and page 9, lines 30 - 32 of the disclosure). PEP is

not consumed in the PTS pathway and can be redirected into other metabolic pathways. (See page 3, lines 11 - 20 of the disclosure).

In one embodiment of the invention, the PTS^-/ Glu^+ strains are further transformed with recombinant DNA coding for one or more genes which direct carbon flow into and through the common aromatic pathway. One such gene is transketolase (*tkt*A or *tkt*B). Transketolase catalyses two separate reaction each of which produces erythrose-4-phosphate (E4P) as a product. (See page 10, lines 26 - 30 and examples 6 - 8 of the disclosure). Example 6, illustrates that production of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) in the PTS^-/ Glu^+ strains obtained according to the invention, is twice as much as the isogenic control. Example 7, illustrates that production of the amino acids phenylalanine and tyrosine is enhanced significantly in the PTS^-/ Glu^+ strains obtained according to the invention when compared to their isogenic controls. Example 8, illustrates that production of the amino acid tryptophan in the PTS^-/ Glu^+ strains, obtained according to the invention was greater than 4 times the production of tryptophan in their isogenic controls.

6. STATEMENT OF THE ISSUES FOR REVIEW

The final rejection presents two issues for review.

1. Whether claims 23, 27, 38, 46 and 49 are rendered obvious under 35 U.S.C. §103(a) over the combined disclosures of Saier et al. and Ingrahm et al.

2. Whether claims 23 - 27, 29 - 31, 33 - 38, 42, 44, 46, 49 and 50 are rendered obvious under 35 U.S.C. §103(a) over the combined disclosures of Frost, Holms, Ingrahm et al. and Saier et al.

7. GROUPING OF CLAIMS

Appellants maintain rejected claims 23 - 27, 29 - 31, 33 - 40, 42 - 44 and 46 - 50 do not stand and fall together. The claims are grouped and argued in three groups A, B and C as follows:

A. Claims 23 - 26, which are directed to a mutant host cell,

B. Claims 38, 46 and 49, which are directed to a method of culturing a mutant host cell under continuous culture conditions, and

C. Claims 27, 29 - 31, 33 - 37, 42, 44 and 50 which are directed to a method.

Reasons as to why appellants consider the rejected claims to be separately patentable are presented below in section 8.B.

8. ARGUEMENT

A. Analysis of the cited references:

Saier et al. - "Characterization of constitutive galactose permease mutants in *Salmonella typhimurium*, *J. Bacteriol.* (1973) 113:512 - 514, hereinafter "Saier".

Saier teaches *Salmonella typhimurium*, lacking the PTS genes (*pts*H and *pts*I encoding Enzyme I and HPr, respectively) cannot transport or metabolize glucose. However when these cells are cultured with glucose as the sole carbon source and mutated by use of nitrosoguanidine some cells regain the ability to use glucose, but do not regain Enzyme I or HPr activity. This phenotype is indicated by PTS^-/glu^+. Saier found that the glucose uptake was due to synthesis of a constitutively expressed galactose permease gene (*gal*P) resulting from a mutation in the repressor gene *gal*R. GalP was confirmed to be an active transport system and not dependent on HPr activity. Table 1 of Saier (attached hereto as Appendix 4) shows that the PTS^-/glu^+ mutants have a generation time of 2 hours. This is in contrast to the PTS^-/glu parent strains disclosed in Saier, wherein there was no recorded fermentation of glucose. As agreed by the appellants and the examiner, a generation time of 2 hours translates to a specific growth rate of 0.35 h^{-1}, more precisely, 0.3465 h^{-1}.

Ingrahm et al. - U. S. Patent 5,602,030; hereinafter "Ingrahm".

Ingrahm discloses a PTS phenotype in which glucose transport is not obligatory coupled to PEP production. Ingrahm is specifically directed to PTS host strains transformed with a heterologous DNA encoding a glucose-facilitated diffusion protein (GLF) and a glucokinase.

The movement of glucose by a GLF is not an active transport, but rather a passive diffusional mediated process. There is no disclosure of a PTS^- phenotype nor a PTS^-/glu^+ phenotype requiring galactose permease activity.

Holms , W. - "The central metabolic pathways of *Escherichia coli*: relationship between flux and control at a branch point efficiency of conversion to biomass, and excretion of acetate, In CURRENT TOPICS IN CELLULAR REGULATION 28:69 - 105 (1986) Academic Press NY; hereinafter "Holms".

Holms generally discloses the central metabolic pathways in *E. coli* and the measurement of carbon flux through them. Holmes specifically teaches that PEP within *E. coli* is a major intermediate for a number of metabolic pathways, such as pyruvate synthesis, oxaloacetate synthesis and aromatic amino acid synthesis. Holms further discloses that the PTS system consumes 66% of the produced PEP. It is also disclosed that the amount of PEP channeled into the aromatic amino acid synthetic pathway is only 3.3%; the amount channeled into the oxaloacetate synthetic pathway is 16.2%, and the amount channeled into pyruvate biosynthesis is 14.5%. Appellants illustrated, in Figure 1 (Appendix 3) of the originally filed specification, the percentages as calculated by Holms to describe the amount of PEP channeled into competing pathways. Holms does not teach or suggest a PTS^- host cell with an altered glucose transport system.

Frost - U.S. Patent 5,168,056; hereinafter "Frost".

Frost teaches the amplification of carbon flow into the common aromatic amino acid pathway by increasing the amount of one of the substrates, erythrose-4-phosphate (E4P). In the first committed step of the aromatic pathway, E4P combines with PEP by the action of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase to form the product DAHP. DAHP synthase is encoded by the genes *aro*G, *aro*F or *aro*H. (Reference is made to Figure 1 attached as Appendix 3). To increase the amount of E4P in a host cell a transketolase (*tkt*A) gene is introduced into the cell. Transketolase catalyzes the conversion of fructose 6-P to E4P.

8.B. Errors Assigned and Argument:

8.B.1. Claims 23, 27, 38, 46 and 49 are patentable under 35 U.S.C. §103(a) over the combined disclosures of Saier et al. and Ingrahm et al.

The determination of obviousness is a legal conclusion based on factual evidence derived from an analysis of 1) the scope and content of the prior art, 2) the difference between the prior art and the claimed subject matter, and 3) the level of ordinary skill in the art at the

time the invention was made. (*Stratoflex, Inc v. Aeroquip Corp.*, 218 USPQ 871 (Fed. Cir. 1983)). To establish a *prima facie* case of obviousness the PTO must satisfy the requirement that the prior art relied upon, coupled with the knowledge generally available at the time of the invention, contains some suggestion or incentive that would motivate the skilled artisan to make the claimed invention. The expectation of success as well as the teaching or suggestion must come from the prior art reference and not applicant's disclosure (*In re Vaeck* 20 USPQ2d 1438 (Fed. Cir. 1991) and *In re Dow Chemical Co.* 5 USPQ2d 1529 (Fed. Cir. 1988)). Furthermore, there must be a reasonable expectation of success, determined from the vantage point of one skilled in the art at the time the invention was made. The prior art reference or combination of references must teach or suggest all the limitations of the claims. Additionally, secondary considerations such as commercial success, long felt but unresolved needs, failure of others may be utilized to give light to the circumstance surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or non-obviousness these inquiries may have relevancy (*Graham v. John Deere Co.*, 148 USPQ 459 (1966). While obviousness does not require absolute predictability, the prior art reference must provide both the suggested modification and a reasonable expectation of success (*In re Dow Chemical Co.*, 5 USPQ2d 1529 (Fed. Cir.1988) and *Amgen Inc v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991)).

8.B.1.(a). Patentability of claim 23.

Claim 23 is directed to mutant host cells which are phenotypically PTS^{-}/ Glu^{+}; require galactose permease activity to transport glucose, and have a specific growth rate on glucose as a sole carbon source of at least 0.4 h^{-1}.

Appellants contend there is no suggestion or motivation provided by either the Saier or the Ingrahm reference when taken alone or in combination which would make the present invention unpatentable. While the Saier reference discloses *Salmonella typhimurium* strains, which are PTS^{-} having a galactose permease transporter and able to grow on glucose, the generation time of these strains is 2 hours. (Reference is made to Table 1 strains SB2637 and SB2634 of Saier attached as Appendix 4). Appellants and the examiner agree that a generation time of 2 hours equals a specific growth rate of 0.35 h^{-1}, more precisely, 0.3465 h^{-1}. Appellants claims are directed to a specific growth rate of at least 0.4 h^{-1}. (Reference is made to page 115 of Biochemical Engineering 2nd Ed. Academic Press, Inc. 1973, New York and

London - submitted as Appendix 5 for the formula to convert generation time to specific growth rate).

There is no suggestion in Saier that a mutant host cell could be produced that provides a PTS^-/glu+ phenotype wherein the specific growth rate is at least 0.4 h^{-1}. Moreover, appellants contend there is no expectation of successfully producing a PTS^-/glu^+ mutant cell having a specific growth rate of at least 0.4 h^{-1}. The examiner has stated on page 4 of the office action dated March 26, 2003,

> "As Saier et al. disclose cells with growth rates very close to the claimed rate of at least 0.4/hr one of ordinary skill in the art would have reasonably expected to be able to obtain cells within the scope of the claims."

and further on page 5,

> "Saier et al. teach two mutant strains (SB2637 and SB2634) which meet all limitations of the instant claims except for the recited growth rate limitation .... ".

and page 6,

> "However, even if the growth rate of these mutant strains isolated by Saier et al. is slightly lower than the 0.4/hr limitation recited in the claim, it is certainly sufficiently close that one of ordinary skill in the art would have expected to be able to isolate similar Pts^- /$glucose^+$ cells with growth rates as claimed."

While one skilled in the art might hope to manipulate PTS^-/ Glu^+ cells to have a higher growth rate there is no expectation, as taught in Saier, that one skilled in the art could reasonably expect to obtain a PTS^-/ Glu^+ cell with a growth rate higher than 0.35 h^{-1} and at least 0.4 h^{-1} as presently claimed.

The examiner argues that since Saier only used one significant digit in the numbers reported, a growth rate of 0.4/hr which is equivalent to a generation time of 1.7 hrs would have been reported as 2 hrs. However, this is simply conjecture on the examiner's part. There is no discussion whatsoever about a mutant PTS^-/glu+ cell having a growth rate of at least 0.4 hr^{-1} or higher.

Further, Saier suggests that the selected PTS^-/ Glu^+ mutants used a constitutive galactose permease (GalP) to transport glucose. More specifically, the inhibition of GalP was removed due to a mutation in the gene encoding GalR, an inhibitor of GalP. However, in the present invention the introduction of a *galR* mutation only partially restored the ability of cells to utilize glucose. (See pages 15 and 16 and examples 4 and 5 of the disclosure).

Appellants further contend that the combination of Ingrahm with Saier adds nothing to advance the examiner's argument of obviousness. As stated above, while Ingrahm recognizes a PTS^- host cell can have an alternative pathway for glucose transport, the reference fails to recognize or disclose that the alternative pathway could involve an endogenous active transport system. Instead Ingrahm teaches a PTS^- host engineered to include a nucleic acid encoding a heterologous GLF. This protein is a glucose facilitated diffusional protein. It is generally well known that the mechanism of facilitated diffusion does not involve energy expenditure. With facilitated diffusion a permease or carrier catalyzes the transport of a compound (in this case glucose) across a gradient, and this transport results in the equilibration of glucose concentration on both sides of a membrane. This mechanism of transport is different from active transport. The transport of glucose by a native GalP transport system as claimed by appellants requires the expenditure of metabolic energy, in the form of membrane potential, and glucose is moved across the membrane, inside of a cell, even against a concentration gradient. A diffusion mechanism will not accumulate a solute inside of a cell against a concentration gradint.

Therefore at best, Ingrahm provides motivation to look for alternative pathways for glucose metabolism in PTS^- cells, but clearly does not suggest a PTS^-/ Glu^+ cell requiring galactose permease (GalP) activity. Moreover, appellants assert that around the time the present invention was made several reports indicated that under glucose limited conditions the MglABC transport system was the preferred system for glucose transport (See Appendix 6 - Death and Ferenci, (1993) Res. Microbiol. 144: 529 - 537).

When viewed as a whole, the combination of Saier and Ingrahm fail to suggest to one of ordinary skill in the art a phenotypically PTS^-/ Glu^+ cell, requiring galactose permease activity to transport glucose, and having a specific growth rate on glucose as a sole carbon of at least 0.4 h^{-1}. Further the combination of references lack any suggestion on the likelihood of success in obtaining PTS^-/ Glu^+ cells requiring galactose permease activity and having a specific growth rate on glucose as a sole carbon source of at least 0.4/hr.

8.B.1.(b). Patentability of claims 38, 46 and 49.

As stated above for a *prima facie* case of obviousness to prevail, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art to combine the referenced teachings; there must be a

reasonable expectation of success; and the prior art references must teach or suggest all the claim limitations. (See MPEP 2141.03 - 2143).

The examiner states at pages 6 -7 in the office action dated March 26, 2003,

> "Applicants discussion of the differences in how the mutant cells with increased growth rates on glucose were obtained between Saier et al. (which treated the Pts- cells with nitrosoguanidine and then grew the treated cells on minimal glucose agar) and applicants (which cultured Pts- cells under continuous culture conditions with glucose as the sole carbon source in order that only glucose+ spontaneous mutants would grow) is noted but not persuasive to overcome the rejection as both methods would have been recognized in the art as equivalent alternatives for isolating mutant Pts- cells that would grow on glucose as the sole carbon source."

Appellants incorporate herein the argument presented above for patentability and further contend that exposing cells to a mutagenizing agent such as nitrosoguanidine and then selecting mutants from minimal glucose agar medium is different from a method of using a continuous culture, such as a chemostat, in order to select glucose + revertants of the PTS^- strains having at least a specific growth rate of 0.4 h^{-1} on glucose. A method of using a continuous cell culture mode, such as a chemostat was specifically chosen in the present invention. As stated as page 14 of the instant disclosure,

> "In order to select spontaneous glucose+ revertants of the PTS^- strain (PB11), selection was performed with a chemostat [29]. The experiment was designed to isolate mutants with a specific growth rate of at least 50% of the parental (PTS+) strain (JM101) (see Experimental Procedures section below). By increasing the feed flow rate in the chemostat, mutants were selected with specific growth rates. These growth rates were confirmed in independent experiments for each strain."

While modes of continuous culture, such as use of a chemostat, for growing bacteria may have been known in the art, there is no suggestion or motivation provided in either the Saier or Ingrahm reference alone or in combination that would suggest a method of obtaining PTS^-/glu+, galactose permease requiring mutant cells comprising culturing the mutant host cells under continuous culture conditions using glucose as a carbon source and selecting mutant host cells which grow on glucose at a specific growth rate of at least 0.4h^{-1}. Ingrahm relates to a method of obtaining a recombinant host cell which expresses a heterologous DNA that encodes a glucose facilitated diffusion protein and a hexokinase. This is a very different

means of providing an alternative pathway for glucose uptake than that presently claimed and while Saier discloses a method of obtaining PTS^-/ Glu^+ cells, this is not the method claimed by Appellants. A method of exposing cells to a mutagen and then growing cells in glucose minimal agar, is different than exposing cells to a continuous culture environment wherein the rate of cell division can be varied by adjustment of inflow rates. Instead of using a method capable of causing multiple mutations in the same strain as disclosed by Saier, appellants' purposely choose a continuous culture method which would exploit the natural capability of the cells to generate spontaneous mutations under stressful conditions and would eliminate mutant strains with a growth rate lower than 0.4 h^{-1}.

8.B.1.(c). Patentability of claim 27.

Although the prior art may suggest the desirability of the result attained by an invention and even encourage researchers to undertake work directed toward the result in a promising field, the prior art must also provide specific guidance as to the particular form of the innovation and the method of attaining it (*In re* Jones 21 USPQ2d 1941 (Fed. Cir. 1992)). The Saier reference focuses on mutating a PTS^- cell with nitrosoguanidine to obtain a PTS^-/ Glu^+ cell having a regeneration time of 2 hours wherein the revertant has a mutation in the *gal*R gene, and Ingrahm focuses on a method of increasing PEP production by enhancing GLF activity in a PTS cell. The Ingrahm reference is related to a completely different mode of increasing PEP availability. Appellants suggest there is not only no motivation provided to combine these references, but also even when combined, they do not establish a *prima facie* case of obviousness. Neither reference alone or combined suggests a method of increasing PEP availability to a biosynthetic or metabolic pathway by obtaining a PTS^-/ Glu^+ host cell requiring galactose permease activity to transport glucose and having a specific growth rate of at least $0.4h^{-1}$, wherein the PTS^- phenotype is caused by the deletion or inactivation of one or all of the genes selected from the group of *pts*I, *pts*H and *crr*, and culturing the host cell mutant in the presence of an appropriate carbon source wherein the mutant utilizes PEP as intermediate of metabolism.

8.B. 2. Claims 23 - 27, 29 - 31, 33 - 38, 42, 44, 46, 49 and 50 are patentable under 35 U.S.C. §103(a) over the combined disclosures of Frost, Holms, Ingrahm et al. and Saier et al.

The same law applied to the errors assigned and argument section 8.B.1. above is applied to this section 8.B.2. In short, MPEP 2141.03 - 2143 instructs, to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art to combine the referenced teachings. Second, there must be a reasonable expectation of success, and third, the prior art references must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination must both be found in the prior art and not based on applicant's disclosure (In re Vaeck, 947 F2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)).

It appears, that the references of Frost and Holms are provided to support the examiner's argument that PTS^{-}/glu+ host cells requiring galactose permease activity and having a specific growth rate of at least 0.4 h^{-1} and further comprising (i) recombinant DNA encoding enzymes such as transketolase, transaldolase and pyruvate synthase or (ii) mutations in genes encoding pyruvate kinase as methods of making said cells, are unpatentable. However, claims 38, 46 and 49 do not recite the additional elements of (i) or (ii). Nevertheless, appellants contend the supplementary references of Frost and Holms add nothing to the already deficient primary references.

8.B.2. (a). - Patentability of claims 23 - 26.

Appellants incorporate the argument as present under section 8.B.1.(a) above with respect to patentability of claim 23.

The examiner states at page 9 of the office action dated March 26, 2003,

> "The disclosure of Frost of amplification of carbon flow into the common aromatic pathway by increasing the amount of one of the substrates (E4P) for the first committed step of this pathway would suggest to the ordinary skilled artisan the amplification of the other necessary precursor (i.e., PEP) of this enzymatic step as this would assure that neither substrate for this enzyme would be in limiting supply. One of ordinary skill in the art would recognize that the supply of any precursor used by a cellular pathway could be amplified by either increasing the amount of the precursor synthesized (such as done by Frost for E4P) or by preventing the depletion of the precursor by other cellular pathways thereby increasing the amount of the precursor available to be used by the desired pathway."

However, appellants contend that the claimed mutant PTS^{-}/glu^{+} cells, having a specific growth rate of at least 0.4 h^{-1} which may further comprise a recombinant DNA coding for one or more of the enzyme transketolase, transaldolase and phosphoenolpyruvate synthase such that the enzymes are expressed at an enhanced level relative to the wild-type host cell, or further that the host cell may comprise a mutation in the pykA or pykF genes, is not suggested by the reference. The Frost reference merely teaches that carbon flow may be enhanced into the aromatic pathway by introducing into a host a polynucleotide encoding a transketolase to increase the amount of E4P produced from fructose 6-P. As illustrated in Appendix 3 - Figure 1, E4P combines with PEP to form DAHP, the first committed product in the common aromatic pathway. Appellants fail to find a suggestion in Frost as to amplification of PEP for increasing flow into the aromatic pathway.

Holms merely provides that the flux to DAHP from PEP is 3.3% and that the carbon flow from PEP could be improved by preventing PEP use by the PTS pathway. At best, Holms suggests that the conversion of PEP into other intermediates could be improved. While Frost may suggest a means of increasing carbon flow to E4P and Holms may suggest the desirability of improving carbon flow by preventing PEP use by the PTS pathway neither reference alone or in combination suggests the claimed galactose permease activity requiring mutant PTS^{-}/glu^{+} cells having a growth rate of at least 0.4 h^{-1}. Neither the Holms reference nor the Frost reference makes up for the deficiencies of Saier and Ingrahm.

Ingrahm does not even disclose a PTS /glu^{+} phenotype requiring galactose permease activity. Ingrahm merely teaches PTS host strains transformed with a heterologous DNA encoding a glucose-facilitated diffusion protein (GLF) and a glucokinase. As discussed above the passive diffusional mediated GFL transport system is completely different that the active GalP transport system. At best, one of skill in the art reading Ingrahm would be motivated to engineer a host cell to comprise GLF and hexokinase and in particular to use heterologous genes to provide such functions.

Moreover while Saier may disclose a PTS /glu^{+} cell having a generation time of 2 hours (equivalent to a growth rate of 0.35 h^{-1}), there is no suggestion in the reference of PTS /glu^{+} cells having a growth rate of at least 0.4 h^{-1}.

Appellants declare the requirements for establishing a *prima facie* case of obviousness over the combined references has not been meet; not all of the claim elements are taught and clearly there is no reasonable expectation of success provided for by the combination of references. (*In re Jones* 21 USPQ2d 1941 (Fed. Cir. 1992)).

8.B.2.(b). – Patentability of claims 38, 46 and 49.

As discussed above in section 8.B.1.(b), claims 38, 46 and 49 have been rejected over the combined teachings of Saier and Ingrahm. Moreover, the examiner has not specifically indicated how the Frost and Holms references supplement the rejection of said claims. Frost is concerned with increasing the carbon flow into the common aromatic pathway of a host cell by enhancing the amount of E4P. The host cells of Frost include cells having a PTS. Holms merely discloses the measurement of carbon flux channeled from PEP into the PTS pathway, the common aromatic pathway and into the oxaloacetate synthetic pathway. Appellants illustrated, in Figure 1 of the originally filed specification, the percentages as calculated by Holms to describe the amount of PEP channeled into the competing pathways. (Reference is also made to Appendix 3, modified Figure 1). While the measurement of carbon flux as provided by Holms may suggest to one of ordinary skill in the art it would be advantageous to rechannel some of the carbon lost to the PTS, neither Holms nor Frost suggest a method of obtaining PTS^-/glu^+, galactose permease requiring mutant cells comprising selecting host cells which utilize a PTS, mutating the cell to inactivate the PTS, culturing the mutant host cells under continuous culture conditions using glucose as a carbon source and selecting mutant cells which grow on glucose with a specific growth rate of at least 0.4 h^{-1}. Appellants incorporate herein the argument presented above for patentability and further contend that exposing cells to a mutagenizing agent such as nitrosoguanidine and then selecting mutants from minimal glucose agar medium is different from a method of using a continuous culture, such as a chemostat, in order to select glucose + revertants of the PTS^- strains having at least a specific growth rate of 0.4 h^{-1} on glucose. A method of using continuous culture conditions was specifically chosen in the present invention.

8.B.2.(c). - Patentability of claims 27, 29 - 31, 33 - 37, 42, 44 and 50.

These claims are directed to a method for increasing PEP availability to a metabolic pathway in a host cell (independent claim 27 and claims 29 – 31 dependent thereon) and to a method of enhancing production of a desired compound in a modified host cell (independent claim 42 and claims 33 – 37, 44 and 50 dependent thereon).

The patentability of claim 27 over the Saier and Ingrahm references has been discussed hereinabove in section 8.B.1.(c), and the discussion is incorporated herein. Moreover, the Frost reference has no teaching or suggestion concerning a method of increasing PEP availability in

a host cell modified to have a PTS^{-}/glu^{+} phenotype nor any disclosure of the need to utilize such as modified host cell. At best, the skilled artisan following the teaching of Frost would be motivated to overexpress the transketolase gene (tkt) in a host cell as a means to increase carbon flow into the aromatic pathway. The claimed method is not just directed to a host cell with increased PEP availability but directed to a method of increasing PEP availability by obtaining a PTS^{-}/glu+ cell requiring galactose permease activity and having a specific growth rate of at least 0.4 h^{-1}. The mere fact that Frost discloses a method of modifying a host cell to increase E4P adds nothing to the deficiency of the primary references in the first place and secondly does not even provide motivation for increasing PEP availability. Furthermore, appellants submit there is no suggestion in Frost to combine the teachings of Frost with art related to phosphotransferase transport systems such as Saier or with any of the cited art.

9. SUMMARY

As stated in M.P.E.P. Section 2141 when applying Section 103, the following tenets of patent law must be adhered to: 1) the claimed invention must be considered as a whole; 2) the references must be considered as a whole and suggest the desirability of making the combination; 3) the references must be viewed without the benefit of impermissible hindsight; and 4) reasonable expectation of success is the standard with which obviousness is determined. Further in determining the differences between the prior art and the claims, the question under Section 103 is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. *Stratoflex, Inc. v. Aeroquip Corp.*, 218 USPQ 871 (Fed. Cir. 1983). Furthermore, as stated in M.P.E.P. 706.02(j), in addition to providing motivation and a reasonable expectation of success, in order to meet the standard for obviousness, all claim limitations must be suggested by the prior art.

Appellants submit that when viewed in its entirety, the presently claimed invention, i.e. claims of group A, direct to host cells being phenotypically Pts-/glu+, requiring galactose permease activity to transport glucose; and having a specific growth rate on glucose as a sole carbon source of at least about 0.4h^{-1}; claims of group B, directed to a method for obtaining the claim host cells comprising culturing the Pts-/glu+ cells under continuous culture conditions; and claims of group C, directed to a method of increasing PEP availability and enhanced

production of a desired compound would not have been obvious in view of any of the references taken alone or in combination.

In summary, Appellants submit that the presently claimed invention is non-obvious in view of the cited references because: 1. there was no motivation provided by any of the cited references alone or in combination to produce the claimed invention; 2. there was no expectation of successfully producing the claimed invention provided by the cited art; and 3. there was no suggestion of the claimed invention as a whole in any of the cited references or suggestion or incentive supporting the combination of references.

As prescribed by 37 CFR §1.192(c)(7), the claims on appeal are found in the attached Appendix 1. As prescribed by 37 CFR §1.192(a), this Brief on Appeal is submitted in triplicate.

An early decision on the merits is kindly solicited.

Respectfully submitted,

Date: August 24, 2003

Lynn Marcus-Wyner, Ph.D.
Registration No. 34,869

Genencor International, Inc.
925 Page Mill Road
Palo Alto, CA 94304
Tel. No.: 650-846-7620
Fax. No.: 650-845-6504

APPENDIX 1

PENDING REJECTED CLAIMS

23. (Previously amended): A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:

(a) being phenotypically Pts-/glu+ wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of a gene selected from the group consisting of *ptsI*, *ptsH* and *crr*;

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of at least $0.4h^{-1}$.

24. (Original): A mutant host cell of Claim 23 comprising recombinant DNA coding for one or more of the enzymes selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase such that the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

25. (Original): A mutant host cell of Claim 23 further comprising mutations in the pykA and/or pykF genes in said host cell.

26. (Original): A mutant host cell of Claim 24 further comprising mutations in the pykA and/or pykF genes in said host cell.

27. (Previously amended): A method for increasing PEP availability to a biosynthetic or metabolic pathway of a host cell, the method comprising,

a) obtaining a host cell mutant characterized by having a Pts-/glu+ phenotype requiring galactose permease activity to transport glucose; and having a specific growth rate on glucose

as a sole carbon source of at least $0.4h^{-1}$ wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of one of the genes selected from the group consisting of *ptsI*, *ptsH* and *crr*; and

b) culturing the host cell mutant in the presence of an appropriate carbon source, wherein said host cell mutant utilizes PEP as a precursor or intermediate of metabolism.

29. (Previously amended): A method of Claim 27 further comprising modifying the host cell mutant to introduce therein recombinant DNA coding one or more of the enzymes selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase such that the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

30. (Previously amended): The method of Claim 27 further comprising modifying the host cell mutant to reduce or eliminate pyruvate kinase activity in said host cell.

31. (Original): A method of Claim 30 wherein pyruvate kinase activity is reduced or eliminated in the host cell by introducing a mutation in DNA encoding one or more of the sequences coding for pyruvate kinase, pyruvate kinase promoter region and other regulatory sequences controlling expression of pyruvate kinase.

33. (Previously amended): A method of Claim 42 wherein the DNA used to transform the host cell encodes one or more enzyme(s) selected from the group consisting of DAHP synthase, DHQ synthase, DHQ dehydratase, shikimate dehydrogenase, shikimate kinase, EPSP synthase and chorismate synthase.

34. (Previously amended): A method of Claim 42 further comprising transforming the host cell with recombinant DNA coding one or more enzyme(s) selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase so that said enzyme is expressed at enhanced levels relative to wild-type host cells.

35. (Original): A method of Claim 33 further comprising transforming the host cell with recombinant DNA coding one or more enzyme(s) selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase so that said enzyme is expressed at enhanced levels relative to wild-type host cells.

36. (Previously amended): A method of Claim 42 wherein the desired compound is selected from the group consisting of tryptophan, tyrosine and phenylalanine.

37. (Original): A method of Claim 36 wherein the desired compound is tryptophan and the host cell is transformed with DNA coding one or more gene(s) selected from the group consisting of *aroG, aroA, aroC, aroB, aroL, aroE, trpE, trpD, trpC, trpB, trpA* and *tktA* or *tktB*.

38. (Previously amended): A method for obtaining a Pts-/Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell under continuous culture conditions using glucose as a carbon source; and

(d) selecting mutant host cells which grow on glucose at a specific growth rate of at least $0.4h^{-1}$.

42. (Previously amended): A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,

(a) obtaining a modified host cell, wherein said modified host cell is characterized by having

(i) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) having a specific growth rate on glucose as a sole carbon source of at least about $0.4h^{-1}$; and

(iv) utilizing PEP as a precursor or intermediate of metabolism,

said modified host cell further comprising recombinant DNA encoding one or more enzyme(s) catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell; and

(b) culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell.

44. (Previously added): The method of Claim 42 wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of one or more gene(s) selected from the group consisting of ptsI, ptsH and crr.

46. (Currently amended): The method of Claim 38 wherein said inactivating is by deleting part or all of gene(s) selected from the group consisting of ptsI, ptsH and crr.

49. (Previously added): The method of Claim 38, wherein the selected mutant host cell has a specific growth rate of at least 50% of the host cell of step a).

50. (Previously added): The method of Claim 42 further comprising recovering said desired compound.

APPENDIX 2

PENDING ALLOWED CLAIMS

39. (Allowed): A method for obtaining a Pts-/Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell using glucose as a carbon source; and

(d) selecting mutant host cells having a specific growth rate on glucose of about $0.8h^{-1}$.

40. (Allowed): A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:

(a) being phenotypically Pts-/Glu$^+$;

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$.

43. (Allowed): A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,

(a) obtaining a modified host cell, said modified host cell characterized by having

(i) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$ and

(iv) utilizing PEP as a precursor or intermediate of metabolism, said modified host cell further comprising recombinant DNA encoding one or more enzymes catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell and

(b) culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell.

47. (Allowed): The mutant host cell of claim 40 further comprising mutations in a gene selected from the group *pykA* and *pykF*.

48. (Allowed): The mutant host cell of claim 40 further comprising recombinant DNA coding for one or more of the enzymes selected from the group consisting of transketolase, transaldolase, and phosphoenolpyruvate synthase wherein the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

51. (Allowed): The method of Claim 43 further comprising recovering said desired compound.

APPENDIX 3

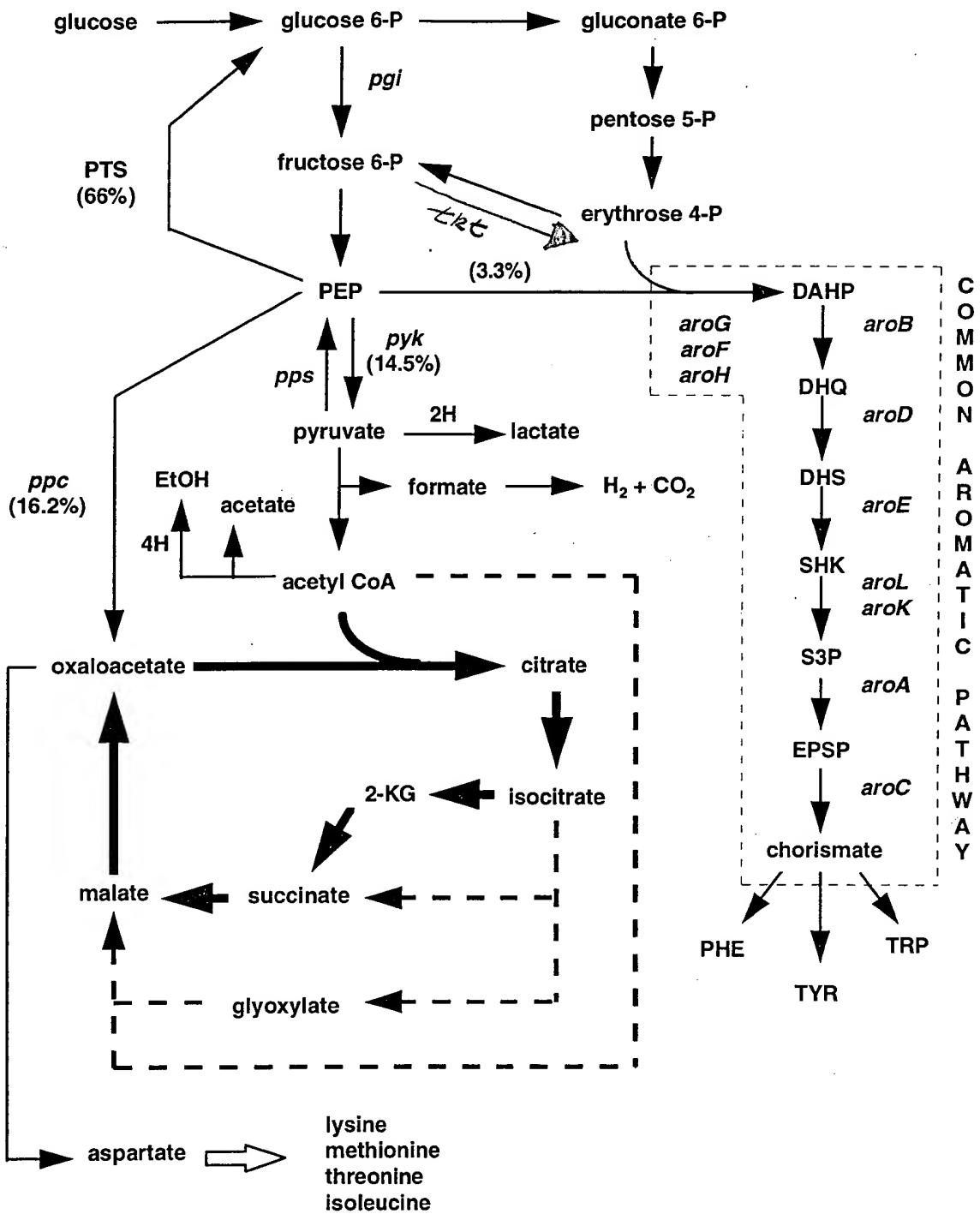
glucose
glucose 6-P
gluconate 6-P
pgi
pentose 5-P
PTS
(66%)
fructose 6-P
tkt
erythrose 4-P
(3.3%)
PEP
DAHP
aroG
aroF
aroH
aroB
DHQ
aroD
DHS
aroE
SHK
aroL
aroK
S3P
aroA
EPSP
aroC
chorismate
COMMON AROMATIC PATHWAY
PHE
TYR
TRP
pps
pyk
(14.5%)
pyruvate
2H
lactate
ppc
(16.2%)
EtOH
acetate
formate
H2 + CO2
4H
acetyl CoA
oxaloacetate
citrate
2-KG
isocitrate
malate
succinate
glyoxylate
aspartate
lysine
methionine
threonine
isoleucine

Fig. 1

APPENDIX 4

JOURNAL OF BACTERIOLOGY, Jan. 1973, p. 512-514

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Characterization of Constitutive Galactose Permease Mutants in *Salmonella typhimurium*

MILTON H. SAIER, JR.,[1] FRED G. BROMBERG, AND SAUL ROSEMAN

Department of Biology and The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

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Salmonella typhimurium strains, lacking both enzyme I and the phosphocarrier protein, HPr, of the phosphoenolpyruvate-sugar phosphotransferase system, cannot transport or metabolize glucose and other sugar substrates of this enzyme system. Mutants which regain the ability to specifically utilize glucose were found to constitutively synthesize a galactose permease by virtue of a mutation in the *galR* gene. This permease, shown to be an active transport system, does not require HPr or enzyme I for activity.

The phosphoenolpyruvate-sugar phosphotransferase system (PTS) catalyzes the translocation of several sugars (PTS sugars) into bacterial cells (7). In *Escherichia coli* and *Salmonella typhimurium* these sugars (of the D configuration) include glucose, fructose, mannose, glucosamine, *N*-acetylglucosamine, hexitols, and β-glucosides. Enzyme components of the PTS include enzyme I and the phosphate carrier protein, HPr, which are required for the phosphorylation of all PTS sugars, and the sugar-specific enzyme II complexes. Mutants lacking enzyme I and HPr cannot utilize PTS sugars for growth. However, these cells can be mutated so that they regain the ability to utilize one or a few PTS sugars without regaining PTS function. Such "pseudorevertants" specifically utilize glucose, fructose, mannose, both fructose and mannose, or *N*-acetylglucosamine (*unpublished results* and references 11 and 14). This communication shows that the mutations permitting glucose utilization result in the constitutive synthesis of a galactose permease which can transport glucose.

The two parental strains used were deleted for the genes which code for enzyme I and HPr (3). Each strain was plated on minimal glucose agar medium (containing the auxotrophic requirement, tryptophan) and was mutagenized with nitrosoguanidine. Mutants were selected which grew on glucose as the sole source of carbon (strains SB2637 and SB2634 in Table 1). [^{14}C]Glucose was taken up by these strains four to six times more rapidly than by the parental strains, and methyl α-glucoside, a nonmetabolizable glucose analogue, was accumulated two- to threefold over the external concentration (Table 1, columns C and D). These strains did not regain enzyme I or HPr activity and showed no alteration in the levels of either glucokinase (adenosine triphosphate [ATP]-D-glucose 6-phosphotransferase, EC 2.7.1.2) or the membrane-associated glucose-specific enzyme II (11).

Enzyme I mutants have been shown to grow on glucose after induction of a galactose permease (1, 12). The mutants described here were therefore tested for galactose uptake and were found to take up this sugar without induction at rates comparable to the fully induced parental strains (Table 1, column E). By contrast, neither methyl β-galactoside (column F) nor thiomethyl β-galactoside transport activities differed in the parental and mutant strains. These results suggest that the constitutive transport system was the previously described "galactose-specific" permease, and not one of several other transport systems which take up galactose (6, 8).

Three types of experiments were performed to further characterize the constitutive galactose transport system. (i) In *E. coli*, the galactose-specific transport system is thought to be regulated by the *galR* gene, which also regulates synthesis of galactokinase (ATP-D-galactose 1-phosphotransferase, EC 2.7.1.6) (2, 9). The mutants isolated here showed the properties expected for *galR* mutants, i.e., the constitutive levels of galactokinase in the mutants were comparable to the fully induced levels in the parental strains (Table 1, column G). In addition, as in the case of the galR gene in *E. coli* (2,

[1] Present address: Department of Biology, University of California at San Diego, La Jolla, Calif. 92037.

TABLE 1. *Properties of transport mutants of Salmonella typhimurium*

Strain No.	Genotype	Glucose fermentation[a]	Generation time on glucose[b] (hrs)	Relative rate of glucose uptake[c]		Relative methyl α-glucoside uptake[d]	Relative rate of galactose uptake[e]		Relative methyl β-galactoside uptake[f]		Relative galactokinase activity[g]	
				Uninduced	Induced		Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
SB2309	*trpB223 ptsΔHI41*	–	7	≡ 1.0	5	≡ 1.0	1	8	≡ 1	14	≡ 1	5
SB2637	*trpB223 ptsΔHI41 galR106*	+	2	6	4	1.9	8	7	1	11	12	10
SB2950	*trpB223 ptsΔHIcrr49*	–	6	1.5	5	ND[h]	1	7	1	32	1	5
SB2634	*trpB223 ptsΔHIcrr49 galR103*	+	2	6	7	ND	10	10	1	5	9	5

[a] Fermentation was conducted with eosin-methylene blue agar without lactose (BBL, Baltimore, Md.) containing 1% glucose. Fermentation was recorded for individual clones after 48 hr at 37 C. Symbols: +, green sheen (positive fermentation); –, pink clones (negative fermentation).

[b] Growth in medium 63 containing 0.2% glucose and tryptophan (20 μg/ml) was conducted and followed as described previously (10).

[c] Cells were grown in nutrient broth (uninduced), or in nutrient broth containing 0.5% galactose (induced), harvested, washed twice with medium 63, and resuspended in medium 63 to a cell density of 0.18 mg (dry weight) per ml. The cell suspensions were shaken at 32 C before addition of [^{14}C]glucose to a final concentration of 1 mM. The rate of uptake of the radioactive sugar, corresponding to the value of 1 in the table, was 1.5 μmoles of sugar per min per g (dry weight) of cells. The rate of uptake was constant for at least 16 min.

[d] Cells were grown in nutrient broth and prepared for the transport experiment as described above. The value of 1 corresponds to an intracellular-to-extracellular concentration ratio of 1.3 after a 16-min incubation period at 32 C with radioactive methyl α-glucoside (20 μM). This value was calculated assuming an intracellular volume of 3 ml/g (dry weight) of cells.

[e] Experiments were performed as in footnote C except that [^{14}C]galactose replaced [^{14}C]glucose in the uptake experiment, and chloramphenicol (50 μg/ml) was included in the transport medium. The value of 1 corresponds to an uptake rate of 3 μmoles per min per g (dry weight) of cells.

[f] Cells were grown to the stationary phase of growth in medium 63 containing 1% potassium lactate and tryptophan (20 μg/ml) (uninduced), or in this medium containing 0.2% galactose (induced). These were diluted with 20 volumes of the same medium and permitted to grow at 37 C for 4 hr before harvesting, washing twice, and resuspending the cells in medium 63 to a cell density of 0.16 mg (dry weight) per ml. The transport experiments were conducted at 32 C in the presence of chloramphenicol (50 μg/ml) and 0.1 mM [*methyl*-^{14}C]β-galactoside. The value of 1.0 corresponds to an intracellular-to-extracellular concentration ratio of about 1.5, 16 min after the addition of the radioactive sugar.

[g] Cells were grown, harvested, and washed as for the experiment described in footnote c. Extracts were prepared by passage of the cells, suspended in 0.02 M potassium phosphate buffer (pH 7.4) plus 1 mM dithiothreitol, through a French pressure cell. Particulate material was removed by centrifugation (50,000 × *g* for 2 min), and galactokinase was determined by a described assay procedure (11) where the concentration of [^{14}C]galactose was 2 mM and the concentration of ATP was 5 mM. Relative rates were not affected by dialyzing the extracts against buffer for 12 hr. Protein was determined by the biuret method (4). The value of 1.0 corresponds to a specific activity of 50 nmoles of galactose phosphorylated per min per mg of protein at 37 C.

[h] Not determined.

9), the mutations permitting glucose utilization were about 95% cotransducible with the *lys* genes and about 60% cotransducible with the *thy* gene, using phage P-22 *int-4* as carrier (*data not shown*). (ii) Strain SB2634 was mutagenized with 2-aminopurine, and galactose-negative mutants were isolated. One such mutant, strain SB2742 (*trpB223 ptsΔHIcrr49 galR103 galK108*), could not phosphorylate or utilize galactose, but it accumulated labeled galactose at least 10-fold over the external concentration (Fig. 1). The intracellular radioactivity was at least 90% free galactose as revealed by chromatographic methods. (iii) Preliminary experiments suggested that the constitutive galactose transport system can transport glucose, mannose, glucosamine, and methyl α-glucoside, but not *N*-acetylglucosamine, fructose, or methyl β-galactoside. These results are in accord with inhibition studies published previously (8).

The simple procedure described here permits the isolation of *galR* mutants and should be applicable to the selection of operator constitutive galactose transport mutants. Our results show that the galactose permease is an active transport system, not dependent on HPr or enzyme I for activity (see also reference 5). The results further support the conclusion (1, 12)

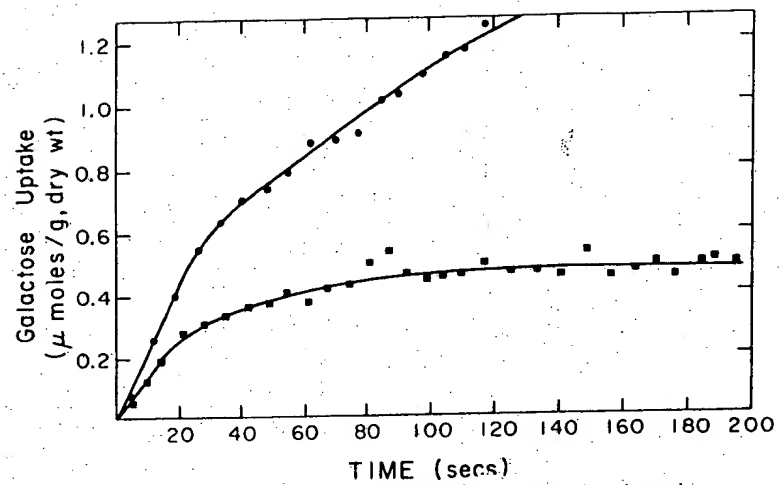

FIG. 1. *Uptake of [^{14}C]galactose by strains which constitutively synthesize the galactose permease. Cells were grown in medium 63 (2) containing 1% potassium lactate as carbon source and tryptophan (20 μg/ml) as growth supplement. They were harvested in the exponential phase of growth, washed once with medium 63, and resuspended in the same medium to a cell density of 2 mg (dry weight) per ml. Transport was followed as described previously (13). One volume of cell suspension, previously brought to temperature equilibration at 24 C, and one volume of medium 63 containing 20 μM [^{14}C]galactose (5 × 10^3 counts per min per μmole, also at 24 C), were mixed at time zero in a stopped-flow apparatus. Subsequently, samples were periodically removed for determination of intracellular radioactivity as described (13). Data are included for strain SB2634, ● (genotype: trpB223 ptsΔ HIcrr49 galR103), and strain SB2742, ■ (genotype: trpB223 ptsΔ HIcrr49 galR103 galK108).*

that poor utilization of glucose by *pts* mutants results from defective transport rather than from a metabolic block. The enzyme II complexes of the PTS evidently cannot catalyze transport unless concomitant phosphorylation of the substrate can occur.

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APPENDIX 5

BIOCHEMICAL ENGINEERING

Second Edition

By

Shuichi AIBA
University of Tokyo

Arthur E. HUMPHREY
University of Pennsylvania

Nancy F. MILLIS
University of Melbourne

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and product formation. In the case of unicellular growth, the growth rate of cells can be expressed in terms of the cell concentration, X, the concentrations of a growth-limiting substrate, S, (Note: there are cases where more than a single substrate can be growth limiting) and an inhibitor, I, i.e.,

$$\frac{dX}{dt} = f(X, S, I) \tag{4.36}$$

Generally, the inhibitor, I, in Eq. (4.36) may imply a predator, X_2 (see Section 4.5 later). In many instances the variables, X, S, and I are highly coupled. Hence, expressions for cell growth rate are usually highly nonlinear.

The specific growth rate, μ, is defined as

$$\mu \equiv \frac{1}{X}\frac{dX}{dt} \tag{4.37}$$

If the value of μ is constant, Eq. (4.37) represents the so-called exponential growth, where growth is proportional to the mass of cells present. Note that the specific growth rate is related to the mass-doubling time, t_d, by

$$t_d = 0.693/\mu = \frac{\ln 2}{\mu} \tag{4.38}$$

Growth other than the exponential type has been proposed. For example, linear growth (dX/dt=constant) occurs in some hydrocarbon fermentations where limitation is due to the rate of diffusion of substrate from oil droplets, provided their surface area is constant.[13,14] In filamentous organisms where growth occurs from the tip, but nutrients diffuse throughout the filamentous cell mass, the growth rate may be proportional to the surface area of mycelia or the 2/3rds power of the cell mass.

4.4.1. Expressions for μ

The following empirical equation has been commonly used to express the specific growth rate, μ,

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{4.39}[28]$$

where

μ_{max} = maximum specific growth rate
K_s = saturation constant
S = concentration of growth-limiting substrate

Equation (4.39) is analogous to Eq. (4.3) (Michaelis-Menten equation), but Eq. (4.39), the so-called Monod equation, has been derived empirically, while Eq. (4.3) is theoretical.

APPENDIX 6

Res. Microbiol.
1993, 144, 529-537

The importance of the binding-protein-dependent Mgl system to the transport of glucose in *Escherichia coli* growing on low sugar concentrations

A. Death and T. Ferenci (*)

Department of Microbiology G08, University of Sydney, N.S.W. 2006 (Australia)

SUMMARY

Glucose limitation in chemostats derepressed the binding-protein-dependent Mgl transport system, which is strongly repressed during growth in batch culture with high glucose levels. The limitation-induced Mgl activity was higher than that of batch cultures "fully induced" for the Mgl system after growth on glycerol plus fucose. Mgl^- strains were impaired compared to Mgl^+ bacteria in removing glucose from sugar-limited chemostats and were outcompeted in mixed continuous culture on limiting glucose. The influence of Mgl was not observed on growth with limiting maltose or non-carbohydrates, and thus was specific for glucose, a known substrate of the Mgl system. In the absence of the two glucose-specific membrane components of the phosphoenolpyruvate:sugar phosphotransferase system, non-PTS-dependent growth on glucose was observed in continuous culture, but only under sugar-limited conditions derepressing the Mgl system and not in glucose-rich batches or continuous culture. Hence growth of *Escherichia coli* on glucose at micromolar concentrations involves a significant contribution of a binding-protein-dependent transport system. The participation of multiple transporters in glucose transport can account for the complex non-hyperbolic dependence of growth-rate on glucose concentration and for discrepancies in studies attempting to describe growth on glucose purely in terms of phosphotransferase kinetics.

Key-words: Glucose, Mgl, Porin, PTS; Galactose-binding protein, Glucose phosphotransferase, Nutrient stress, Starvation response, *E. coli*.

INTRODUCTION

Recently obtained evidence suggested that the full range of transport proteins involved in glucose uptake into *Escherichia coli* was not fully recognized (Death *et al.*, 1993), even though the study of glucose transport has a long history.

As discussed in this study, sugar-specific periplasmic as well as outer membrane proteins contribute to transport, particularly under growth conditions involving limiting, micromolar extracellular glucose concentrations. These systems operate in addition to the phosphoenolpyruvate:sugar phosphotransferase system (PTS)

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(*) To whom correspondence should be addressed.

whose role in growth on glucose in batch culture is well established (Curtis and Epstein, 1975; Postma and Lengeler, 1985; Erni, 1989).

The PTS is involved in both transport and phosphorylation across the cytoplasmic membrane (Postma and Lengeler, 1985). There are two PTS recognizing glucose with different affinities, with the PtsG system having a greater transport affinity (apparent K_m 10-20 μM) than the PtsM (apparent $K_m \simeq 1.3$ mM, Hunter and Kornberg, 1979) for glucose. Phosphotransferase activity is present in bacteria growing in continuous culture at limiting glucose concentrations (Hunter and Kornberg, 1979). Mutants lacking both glucose-specific components were greatly impaired in growth on glucose and thus no other transport system can replace these components, at least under batch or plate-culture conditions (Curtis and Epstein, 1975).

Suspected but less well established is the role of the Mgl transport system in glucose uptake. The Mgl system was initially identified as a high-affinity galactose transport system (Ganesan and Rotman, 1965; Kalckar, 1971; Lengeler *et al.*, 1971). Mgl has a high affinity for both galactose and glucose via its periplasmic glucose/galactose binding protein, whose structure in the presence of ligands is well-defined and which has a K_d of 0.2 μM for glucose (Vyas *et al.*, 1991). Recently, a clue to the involvement of Mgl in glucose transport was the observation that PTS enzyme II mutants of *Salmonella typhimurium* in glucose-limited chemostats accumulated *mgl*-constitutive mutations (Ruiter *et al.*, 1990). The Mgl system can transport glucose but is strongly catabolite-repressible by glucose (Benner-Luger and Boos, 1988; Henderson *et al.*, 1977; Henderson, 1980) so its contribution to growth on this sugar would appear paradoxical. Mgl was always thought to be more physiologically relevant to the transport of galactose, and the *mglBAC* genes are regulated by the *mglD (galS)* repressor binding the same inducers (galactose, fucose) as the *galR* repressor (Weickert and Adhya, 1992). We show that Mgl is significantly derepressed by the transition from glucose-rich to glucose-limited growth in the absence of exogenous inducers and establish that the Mgl system contributes significantly to the growth and transport affinity for glucose at low extracellular concentrations. Indeed under glucose limitation, Mgl can at least partially supplant the PTS for growth on glucose. Hence Mgl is an important glucose scavenging system in *E. coli* under conditions of growth which the bacterium is more liable to find in its natural habitats (Koch, 1971).

MATERIALS AND METHODS

Bacterial strains

All bacterial strains were derivatives of *E. coli* K12 and are shown in table I. P1 transduction (Miller, 1972) with P1 *cmlclr/1000* grown on LA5731 as donor was used to introduce *mgl*::Tn*10* into BW2900 and BW2901 to construct Mgl^- strains with single amino acid markers. BW2924 was constructed by transducing WK124 to Mgl^- using the transposon.

Growth media and culture conditions

The chemostat and batch medium and culture conditions used in this study are as previously described (Death *et al.*, 1993). The glucose concentration in the glucose-limited feed medium was 0.02 % in minimal medium A (Miller, 1972). Nitrogen-limited chemostats were limited for ammonium sulphate (0.045 g/l instead of 1 g/l) with 0.4 % glucose in the feed medium. For all chemostats, the dilution rate was $D = 0.3\ h^{-1}$ unless otherwise specified.

Determination of glucose concentrations

Chemostats were sampled by on-line filtration of 5-ml culture through a 0.2-μm "Acrodisc" syringe filter (Gelman Sciences). Glucose in the filtered culture fluid (concentrated 20-fold by freeze-drying) was assayed using a commercial glucose oxidase method (Sigma Diagnostics Glucose (Trinder) kit, Sigma

Mgl = methyl galactoside. | PTS = phosphotransferase.

Table I. Bacterial strains used in tis study.

Strain	Genotype	Parent strain	Origin/reference
BW1022	HFrG6 *metA trpE aroB*		Ferenci and Lee (1982)
BW2000	HfrG6 *his mgl*::Tn*10*	HFrG6	Muir *et al.* (1986)
UE26	*glk-7 pts*G2 *pts*M1 *rps*L		W. Boos
WK124	UE26 *glk*$^+$ *zfc-765*::Tn*10*		W. Boos
BW2900	HFrG6 *trpE*	BW1022	This study
BW2901	HFrG6 *metA*	BW1022	This study
BW2920	HfrG6 *trpE mgl*::Tn*10*	BW2900	This study
BW2921	HfrG6 *metA mgl*::Tn*10*	BW2901	This study
BW2924	WK124 *mgl*::Tn*10*	WK124	This study
LA5731	F$^-$ *ptsF lacY arg mgl515 zee 700*::Tn*10* (P1*cmlclr1000*)		W. Boos

Chemical Co., Sydney) according to the manufacturer except that the reaction buffer was supplemented with additional glucose oxidase (2 mg/ml; Sigma Chemical Co., Sydney) to increase the sensitivity of the assay.

Transport assays

Samples (10 ml) from chemostat or batch cultures were harvested, washed twice in MMA and resuspended to an identical optical density (A_{580nm} = 0.2). To start assays, 60-μl of bacterial suspension were added to 12 μl ^{14}C-glucose (0.5 μM final concentration) or 12 μl ^{14}C-galactose (1 μM final concentration) at room temperature (20-25°C). Radioactive sugars were from Amersham (Australia), Sydney. Samples (20 μl) were removed at 10, 20, 30, 40 and 120 s and immediately filtered through 0.45-μM cellulose nitrate membrane filters (Whatman Ltd, Maidstone, England) and washed with 10 ml MMA. The filters were counted in liquid scintillant (Aqueous Counting Scintillant, Amersham, Sydney, Australia) and the initial rate of sugar uptake was calculated from the slope derived from the initial timepoints.

RESULTS AND DISCUSSION

In confirmation of many previous studies on the Mgl system in batch cultures of *E. coli,* the Mgl system is inducible by the non-metabolizable sugar fucose and fully repressed during growth on glucose, as assayed by the transport of micromolar ^{14}C-galactose (fig. 1). At the low concentration in the assay, galactose is almost exclusively transported by the Mgl system and the rate of transport by Mgl$^-$ bacteria is negligible (Kalckar, 1971; also fig. 1A). But wild-type bacteria grown in glucose-limited chemostats can transport galactose at a rate much greater than the same bacteria cultured under glucose-rich conditions (fig. 1A). The Mgl$^-$ strain cultured in glucose-limited chemostats transported galactose at a basal level, indicating that it was indeed the Mgl system contributing to increased transport in glucose-limited cells. Most strikingly, the rate of galactose transport in wild-type bacteria under glucose limitation is higher than its highly induced level in batch culture (glycerol + fucose-grown bacteria, fig. 1B). Therefore, under glucose limitation, the level of Mgl activity could be derepressed to high levels and was largely independent of known exogenous inducers such as galactose or fucose. Glycerol-limited growth in chemostats also results in higher galactose transport than found in glycerol batch culture (fig. 1B), but only marginally so. Glucose limitation was clearly more likely to induce Mgl, reminiscent of the recently observed higher induction of LamB by glucose as against glycerol limitation (Death *et al.,* 1993). Glucose-limited cells are well adapted to scavenge any galactose as well as glucose that becomes available in the glucose-starved environment.

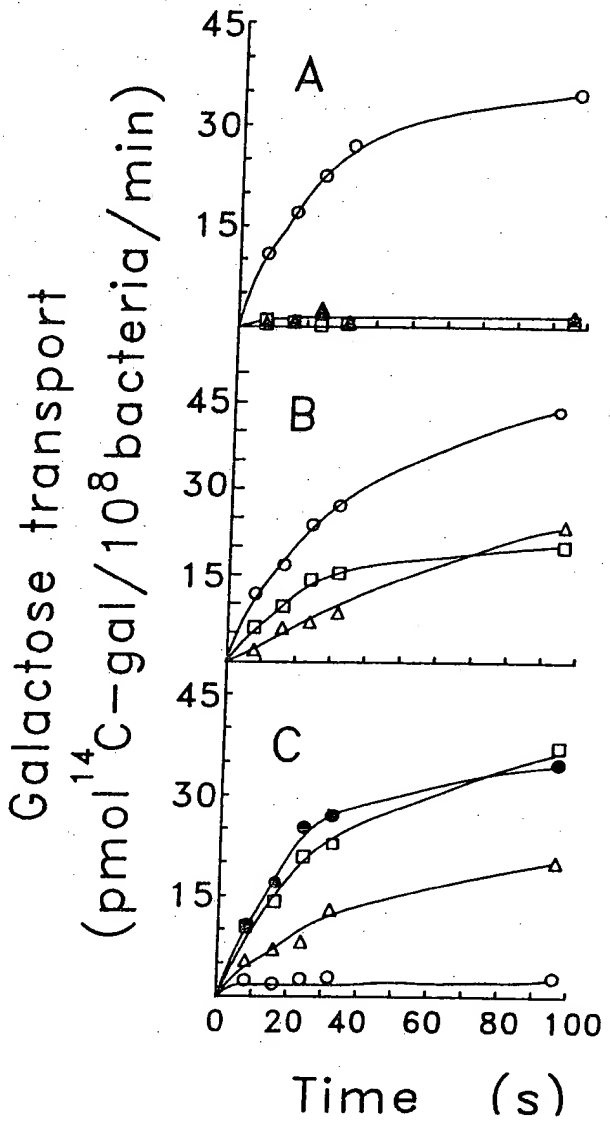

Fig. 1. Galactose transport by wild-type and *mgl* bacteria.

Bacteria were harvested, washed in MMA and the initial rate of uptake was measured as previously described, using ^{14}C-galactose at 1 μM concentration (Death *et al.*, 1993).

A) Wild-type strain BW2901 was grown in glucose-limited chemostat (○) or in batch culture with 0.2 % glucose (▲). The Mgl⁻ mutant BW2921 was grown in glucose-limited chemostat (□).

B) Wild-type strain BW2901 was grown in glycerol-limited chemostat (□) or in batch culture with 0.2 % glycerol (△) or 0.2 % glycerol plus 1 mM fucose (○).

C) Wild-type strain BW2900 was cultivated in a glucose-limited chemostat (●) and, after 48 h, the chemostat bacteria were used as inoculum for overnight batch cultures with 0.2 % glucose (○), 0.2 % glycerol (△) or 0.2 % glycerol plus fucose (□).

The high level of Mgl activity in chemostats after two days of cultivation was not due to the accumulation of *mgl*-constitutive mutants in the culture. Reinoculation of the chemostat culture into high-glucose or high-glycerol batch culture repressed Mgl activity to pre-chemostat levels, although reinoculation into glycerol plus fucose resulted in maintenance of full Mgl induction (fig. 1C).

Given the striking derepression by glucose limitation and the known affinity of the Mgl system for glucose, it became important to determine whether the elevated level of the Mgl system contributed to growth on glucose at low external concentrations. If the Mgl system has a role in glucose transport, then Mgl⁺ and Mgl⁻ strains should show differences in growth affinity towards low concentrations of glucose. The growth affinity of *E. coli* strains for substrate correlates inversely with the residual sugar concentration in chemostats in steady state under glucose limitation (Death *et al.*, 1993). If Mgl contributes to glucose scavenging, then the residual glucose concentration should be higher in glucose-limited continuous culture of Mgl⁻ as against Mgl⁺ bacteria. Chemostats limited by glucose were established for wild-type and the Mgl⁻ mutant, and the steady-state glucose concentration was estimated. Table II shows that the residual glucose concentration for wild-type was 4-fold lower than that measured for the *mgl* mutant. This clearly indicates that the glucose affinity of the Mgl system is contributing to the removal of low concentrations of glucose and that the scavenging ability of wild-type *E. coli* is enhanced by the presence of Mgl.

To further explore the contribution of Mgl to growth on glucose, *mgl* mutants were competed in mixed culture against wild-type *E. coli* to determine the influence of the Mgl system on growth competition at low external concentrations. In these experiments, steady-state cultures of the strains, grown in independent glucose-limited chemostats, were mixed 1:1 for competition in the same medium with growth continuing at the same dilution rate. The degree of competition between the strains was determined by plate counts using different auxotrophic markers in combination with the *mgl* allele. To make

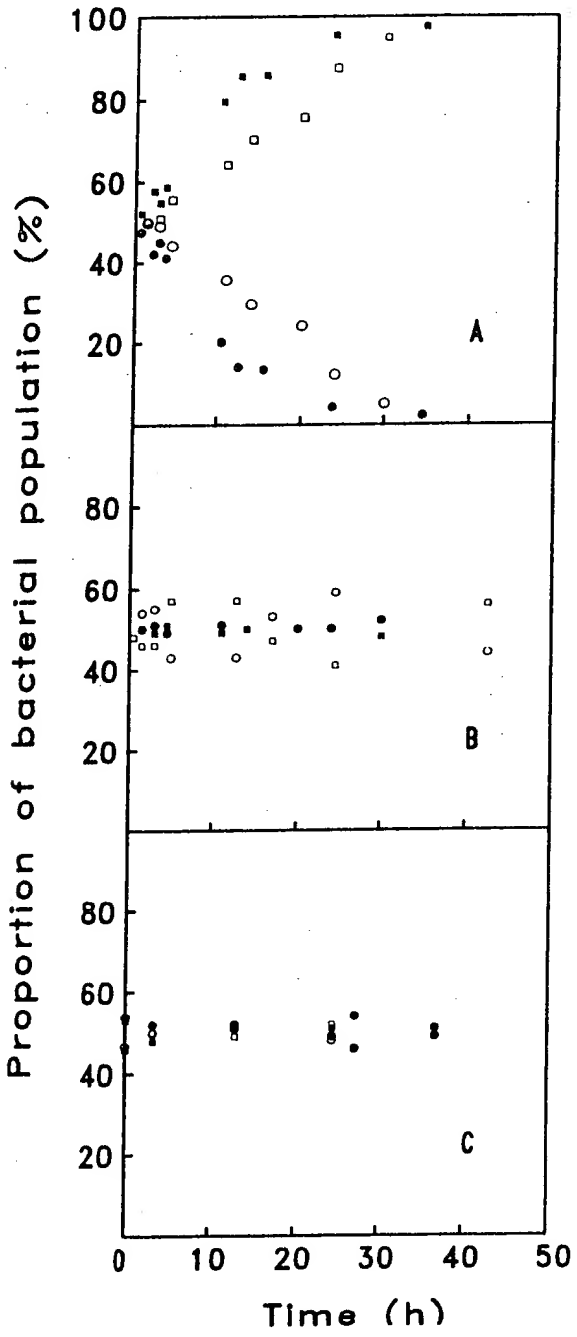

Fig. 2. Competition between Mgl^+ and Mgl^- strains for different limiting nutrients.

Experiments were performed in, (A) glucose-limited (0.02 % carbon source), (B) maltose-limited (0.02 %) and (C) succinate-limited (0.06 %) chemostats. Panels show numbers of bacteria from plate counts in mixed culture containing bacteria with reciprocal pairings of auxotrophic markers and *mgl* allele. For each competition experiment, independent chemostat cultures (D = 0.28 h^{-1}) were established for BW2900(*trpE*, ▩) and BW2921(*metA mgl*, ●) or BW2901(*metA*, □) and BW2920(*trpE mgl*, ○). These cultures were mixed 1:1 to follow competitive growth in the same medium (MMA supplemented with both Trp and Met (40 μg/ml) and carbon source as stated above).

certain that the auxotrophic markers were not influencing growth selection between the strains, experiments were performed with two reciprocal pairings of auxotrophic (*trp* or *met*) and *mgl* markers. As shown in figure 2A, competition for glucose resulted in positive selection of wild type regardless of marker background and the *mgl* mutant strains were washed out. No Mgl-dependent selection was observed with succinate (0.06 %) or maltose (0.02 %) as limiting substrates (fig. 2B and 2C), so the selective advantage of Mgl is restricted to a known substrate of the Mgl system.

These results suggested that the Mgl system was contributing a significant selective advantage to *E. coli* growing at low external concentrations of glucose. Given the positive influence of the Mgl system in growth experiments, it was important to test whether higher glucose transport rates at low external concentrations accounted for the faster growth rate of wild-type bacteria over the *mgl* mutant. The glucose transport rates are also summarized in table II for bacteria in batch and chemostat culture. The general increase in transport rates from batch- to chemostat-grown bacteria even in Mgl^- bacteria is due to the derepression of LamB glycoporin, whose induction under these conditions improves glucose transport kinetics (Death *et al.*, 1993). But significantly, the presence of the Mgl system increases the rate at which *E. coli* can transport low concentrations of glucose. So at submicromolar concentrations, Mgl may be responsible for up to a third of the glucose transported, the rest presumably being taken up via the phosphotransferase system. The proportion of glucose transported via Mgl is likely to be less at higher substrate levels, as Mgl is saturated at micromolar concentrations while PTS is saturated at approximately 10-fold-higher glucose levels. Nevertheless, the significant role of the Mgl

Table II. Glucose scavenging ability and glucose transport rates in Mgl^+ and Mgl^- bacteria.

Strain/allele	Residual chemostat glucose concentration(*) (μM)	0.5 μM ^{14}C-glucose transport rate (pmol/min/10^8 bacteria)	
		Batch culture	Chemostat culture
Wild-type Mgl	3.15 ± 1.1 (4)	23.5 ± 0.67 (6)	61.4 ± 1.9 (6)
Mgl^-	14.25 ± 1.6 (4)	22.20 ± 2.0 (6)	44.6 ± 1.1 (6)

(*) Residual glucose levels in steady state chemostats were measured when the bacteria were growing at a dilution rate of 0.3 h^{-1} with an input glucose concentration of 0.02 % (1.1 mM). The numbers in parentheses represent the number of independent determinations on different chemostats.

system in glucose transport at micromolar concentrations could not have been predicted from previous batch culture experiments. As shown here, strong catabolite repression of the Mgl system under glucose-rich conditions does not reveal the importance of this system under low external glucose concentrations.

Growth on glucose has been largely interpreted in terms of PTS function. But if Mgl is important physiologically when derepressed by nutrient limitation, it should be capable of at least partially replacing the PTS in supplying glucose for growth under these conditions. Hence, a double mutant deficient in glucose-specific enzymes II (PtsG, PtsM) was inoculated into two chemostats, one with glucose limitation and one limited by nitrogen supply but with high, repressing concentrations of glucose. These chemostats were established with glycerol as the initial carbon source, and once equilibrated under carbon or nitrogen limitation, the carbon source was switched to glucose at the same dilution rate. As shown in figure 3B, PTS^- bacteria exposed to high glucose levels (rising for the first 10 h of the experiment due to the switch from glycerol) were washed out, whereas those exposed to limiting glucose (fig. 3A) continued growing, albeit with a somewhat lowered cell density. The Mgl transport system cannot totally replace the glucose transport capacity normally provided by the PTS, as also reinforced by the finding that the steady state residual glucose level with PTS^-Mgl^+ bacteria was much higher (about 0.6 mM) than in cultures of PTS^+Mgl^- bacteria shown in table II. The glucose transport rate of PTS^-Mgl^+ bacteria was also determined after 24 h in the chemostat shown in figure 3A and found to be 27.3 pmol glucose transported/min/10^8 bacteria at 0.5 μM glucose. This rate is less than half the rate of wild-type PTS^+Mgl^+ bacteria (table II), but it is difficult to compare these rates due to the differences in residual glucose levels (and hence expression levels) during growth of the two strains.

As also shown in figure 3C, a PTS^- mutant additionally lacking glucokinase was unable to grow under glucose limitation. This indicated that the glucose entering the PTS^- bacteria in figure 3A required intracellular phosphorylation, consistent with glucose entering via the non-phosphorylative Mgl transporter. In further confirmation, another construct lacking Mgl as well as the PTS systems was also unable to grow on limiting glucose. Hence no other transport system needs to be postulated to explain growth on glucose at low concentrations. These results taken together are consistent with glucose entering via the active Mgl system under glucose limitation conditions but with a lower flux than in the presence of the PTS.

In summary, the Mgl system has a dual function. One role, under nutrient stress at limiting glucose concentrations, is in scavenging glucose. It is worth noting that a protein homologous to the galactose/glucose-binding protein was recognized as having a role in glucose transport in *Agrobacterium* growing in glucose-limited con-

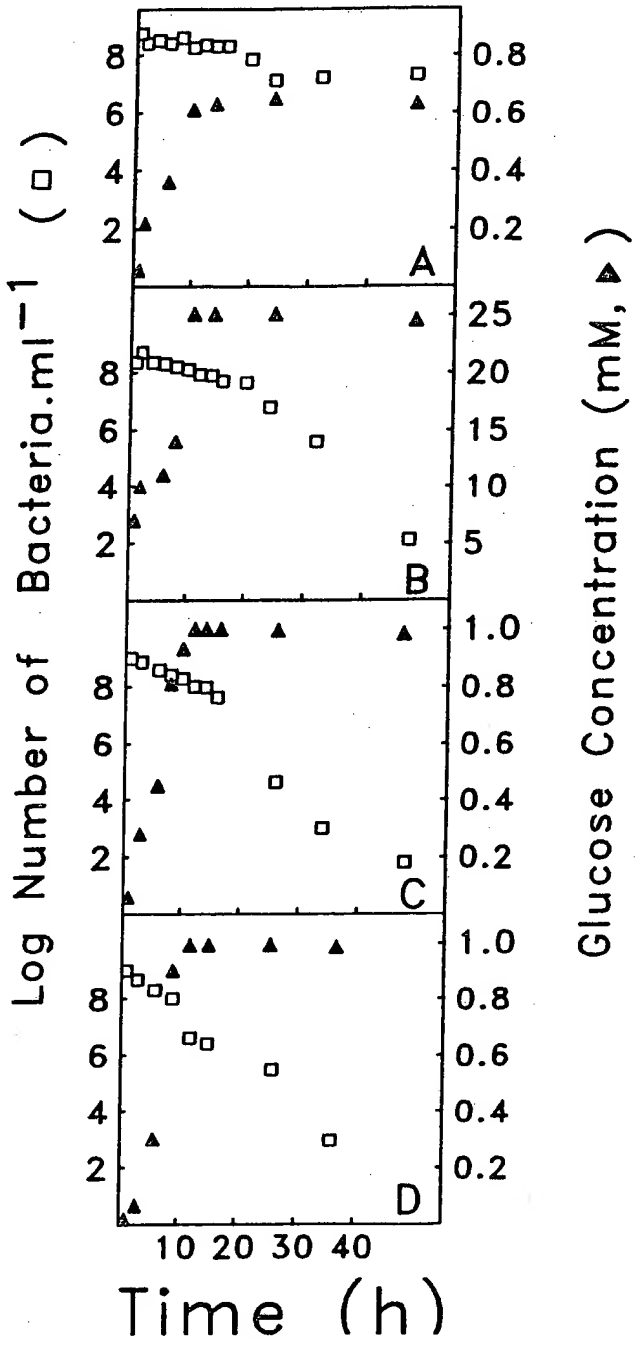

Fig. 3. Growth of PtsG$^-$PtsM$^-$ bacteria on limiting glucose concentrations.

A) The PTS$^-$ double mutant WK124 was grown in a 0.02 % glycerol-limited chemostat at D = 0.28 h^{-1} until at steady state. At zero time, the carbon source in the medium was switched to 0.02 % glucose at the same dilution rate. Bacterial density was followed by viable counts (□) and the glucose concentration in the culture was measured using the glucose oxidase method (▲).

B) The conditions were as described in A, except that initial glycerol concentration was 0.4 % and the chemostat was ammonium-limited (0.045 g/l), with the feed glucose concentration at 0.4 % from zero time.

C) The conditions were identical to A, except that the inoculum was the PTS$^-$Glk$^-$ strain UE26.

D) The conditions were identical to A, except that the inoculum was the PTS$^-$Mgl$^-$ strain BW2924.

tinuous culture (Cornish *et al.*, 1989). The second role of Mgl is in the high-affinity transport of galactose (Kalckar, 1971). But even for galactose uptake, Mgl is less significant than the alternative GalP system at high nutrient levels (*i.e.* in batch culture) as Mgl is more susceptible to catabolite repression (Henderson, 1980). The dual function of Mgl in sugar transport has definite parallels to the recently revealed dual role of the LamB glycoporin. Under nutrient stress, this porin is derepressed in the absence of inducer (maltosaccharide) and contributes significantly to glucose, lactose, arabinose and glycerol permeability across the outer membrane during growth at low sugar concentrations (Death *et al.*, 1993). This is in addition to the previously recognized role of LamB in maltodextrin transport at all concentrations when specifically induced by maltosaccharides (Ferenci and Boos, 1980). A most interesting question is the mechanism of the high-level induction of Mgl under glucose-limited derepression. Mgl activity is very sensitive to cAMP/Crp-dependent activation and the high level of induction in chemostats is *crp*-dependent (Death and Ferenci, unpublished results). But it would be surprising if increased cAMP levels alone under glucose limitation in chemostats (Matin and Matin, 1982; Death and Ferenci, unpublished results) would be enough to account for Mgl activity higher than in fully induced batch cultures, given that the *mgl* system is regulated by a repressor as well as Crp/cAMP (Weickert and Adhya, 1992). A likely explanation is that limitation conditions increase the level of endogenous inducer (galactose), since an intracellular galactose pool of less than 2×10^{-4} M is sufficient for internal induction, and Mgl becomes constitutive in this manner in *galK* mutants (Kalckar, 1971).

Preliminary studies support this possibility with high, inducing levels of galactose found in wild-type bacteria grown in glucose-limited chemostats (Death and Ferenci, unpublished results). As a consequence, the *gal* regulon gene product GalK (galactokinase) is also fully induced under glucose-limited growth conditions. The source of the galactose is as yet unknown and requires further investigation.

The findings in this study should have a considerable impact on the interpretation of a large body of earlier studies on growth kinetics, growth affinities and transport in glucose-limited chemostats and batch cultures. For example, the non-hyperbolic concentration dependence of growth rates of enteric bacteria on glucose (Koch, 1979; Rutgers *et al.*, 1989; Shehata and Marr, 1971) may be due to the contribution of several glucose transporters with different kinetic parameters, including Mgl, PtsG and PtsM. The picture of glucose transport is even more complicated by outer membrane permeability, as glucose permeation is mainly through the saturable glycoporin at low concentrations (Death *et al.*, 1993) but through non-saturable porins at higher concentrations. The exact contributions of all these systems to growth on glucose is not easy to estimate experimentally, and expression of each component (except perhaps PtsG) will vary significantly depending on the external concentration of sugar (Notley, L., Death, A. and Ferenci, T., unpublished observations). Also, studies attempting to correlate the transport activity of enterobacteria growing in glucose-limited continuous culture to phosphotransferase activity (*e.g.* Hunter and Kornberg, 1979; O'Brien *et al.*, 1980; Benthin *et al.*, 1992) are made highly questionable by these results, and such studies would also have been affected by the *mal* and *mgl* genotype and expression of the strains used.

Acknowledgements

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Importance du système Mgl dépendant de la protéine affine dans le transport du glucose chez *Escherichia coli* se développant à des faibles concentrations de glucose

La limitation du glucose en culture continue en chémostat déréprime le système de transport Mgl (méthyle galactoside) dépendant de la protéine affine, système qui est fortement réprimé pendant la croissance classique (en tubes) à des fortes concentrations de glucose. L'activité Mgl induite par la limitation se montre supérieure à celle des cultures en tubes «totalement induite» dans le système Mgl après croissance sur glycérol + fucose. Les souches Mgl$^-$ sont altérées par rapport aux souches Mgl$^+$ (élimination du glucose des chémostats limités en glucose) et sont mises hors compétition en culture continue mixte sur glucose limitant.

L'influence du système Mgl n'est pas observée lors du développement sur maltose limitant ou sur des milieux non glucidiques; ainsi s'est-elle montrée spécifique du glucose qui est un substrat reconnu du Mgl. En l'absence des deux composants membranaires du système phosphotransférase (PTS) spécifiques du glucose, une croissance sur glucose non dépendante du PTS est observée en culture continue mais exclusivement quand le sucre «limite» déréprime le système Mgl et non quand les milieux sont riches en glucose, ou en culture continue. La croissance de *Escherichia coli* à des concentrations micromolaires implique une importante contribution d'un système de transport lié à la protéine affine.

La participation de transporteurs multiples dans le métabolisme du glucose peut expliquer la dépendance complexe non hyperbolique du taux de croissance sur glucose et expliquer les divergences notées dans les descriptions de la croissance sur glucose, exclusivement en termes de cinétique de phosphotransférase.

Mots-clés: Glucose, Mgl, Porine, PTS; Protéine liant le galactose, Glucose phosphotransférase, Stress de nutrition, Réponse aux limitations, *E. coli*.

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